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Modulation of the kinetics of cholesterol side-chain cleavage by an activator and by an inhibitor isolated from the cytosol of the cortex of bovine adrenals*

(steroid sulfates/cytochrome P-450$_{scc}$)

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ABSTRACT **Two modulators of sterol side-chain cleavage activity have been detected in the cytosol from the cortex of bovine adrenals. One is an inhibitor of side-chain cleavage which increases the K_m of a purified and reconstituted mitochondrial side-chain cleavage system for both cholesterol and cholesterol sulfate. It also lowers the V_{max} of cleavage when cholesterol sulfate is the substrate. The other modulator is a low molecular weight protein which in the reconstituted system increases the V_{max} of cleavage for both substrates but does not affect the K_m of either. Side-chain cleavage activity was also found in bovine adrenocortical cytosol and this appears to differ from that present in mitochondria.**

The first step in steroid hormone biosynthesis from sterols is the cleavage of six of the eight carbon atoms that constitute the side chain of cholesterol (Chol). The products are pregnenolone (Preg) and a six-carbon fragment, isocaproic aldehyde. In the adrenal cortex, this conversion is catalyzed by an enzyme system consisting of adrenodoxin, adrenodoxin reductase, a NADPH-generating system, and a cytochrome P-450$_{scc}$ enzyme which can be isolated from the inner mitochondrial membrane.

Over the past 20 years, evidence for the existence of several steroidogenic pathways has accumulated. The extensively documented pathway is that which uses intermediates whose oxygen functions at C-3 are present as hydroxyl groups. Other pathways involve steroidal intermediates esterified at C-3 with either sulfuric or fatty acids. Since 1964 it has been known that cholesterol sulfate (CholSO_4) is metabolized both *in vivo* and *in vitro* to sulfated steroids—pregnenolone sulfate (PregSO_4), 17OH-Preg, and dehydroisoandrosterone sulfate (DHisoAndSO_4)—without cleavage of the sulfate group (1, 2). More recently, several nonpolar short-chain fatty acid esters of Chol were also found to be cleaved to the corresponding Preg derivatives without loss of the ester group at C-3 (3). In addition, long-chain fatty acid esters of Preg have been shown to be metabolized *in vitro* to the corresponding esters of 17OH-Preg and DHisoAnd without cleavage of the ester linkage (4).

The physiological significance of steroidogenic pathways that use substrates of such disparate polarities as acyl esters of Chol, free Chol, or the sulfate of Chol is not known at present, but one possibility is that esterified sterols serve as precursors for steroidal secretory products that are different from those derived from free Chol. Such a relationship between precursor and product could be an integral part of a mechanism that regulates the formation of a specific steroid hormone. The data reported in this paper suggest that this regulation might be influenced by modulatory proteins that control the rate of metabolism of one precursor relative to that of another.

When crude sonicates of bovine adrenal cortical mitochondria are examined, in an assay system, for their ability to cleave the side-chains of Chol and CholSO_4, the kinetics of oxidation of each sterol substrate are biphasic. The unpurified enzyme system exhibits both a low K_m and a high K_m of oxidation for each substrate (5). Such biphasicity is commonly ascribed to either negative cooperativity or substrate activation. In addition, the rates of cleavage of Chol and CholSO_4 at low concentrations are almost additive, suggesting that each substrate is oxidized by its own high-affinity enzyme system. When cytochrome P-450$_{scc}$ is purified (6), the kinetics of oxidation are different from those of the native mitochondria. Upon purification and reconstitution into a side-chain cleavage (scc) enzyme system with adrenodoxin and adrenodoxin reductase, cytochrome P-450$_{scc}$ displays only a low K_m of cleavage for both Chol and CholSO_4. Moreover, rather than activating the enzyme system, high concentrations of either substrate inhibit scc. The purified adrenocortical mitochondrial scc system displays a higher V_{max} for CholSO_4 cleavage than for the cleavage of Chol. This result is opposite to that obtained with sonicates of mitochondria from adrenal cortex or corpora lutea (unpublished data). With these impure preparations, the rate of oxidation of free Chol is higher than that of CholSO_4.

The work reported here originated as a search for regulatory factors which might affect the substrate specificity of the scc system. Our intent was to determine whether factors other than membrane phospholipids were responsible for the differences between the kinetic properties that characterize the crude scc enzyme system of mitochondrial sonicates and those of the purified reconstituted enzyme system. Because proteins that bind Chol and stimulate Preg synthesis by adrenocortical mitochondria have been isolated from the cytosol of several tissues (e.g., liver, adrenal cortex, and corpora lutea) (7–12), a search for factor(s) that affect the activity of a reconstituted mitochondrial scc system was made in cytosol from bovine adrenal cortex. In this communication we report that at least two such modulators are present in the cytosol. In the course of this effort, we have also confirmed earlier reports (13, 14) that there is a scc enzyme present in adrenal cortical cytosol. We have found that the properties of this active material are different from those of the enzyme system found in mitochondria.

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Abbreviations: Chol, cholesterol; CholSO_4, cholesterol sulfate; Preg, pregnenolone, (3β-hydroxy-5-pregnen-20-one); PregSO_4, pregnenolone sulfate; 17OH-Preg, 3β,17-dihydroxy-5-pregnen-20-one; 17OH-PregSO_4, 3β-sulfoxy-17-hydroxy-5-pregnen-20-one; DHisoAnd, dehydroisoandrosterone (3β-hydroxy-5-androsten-17-one); DHisoAndSO_4, dehydroisoandrosterone sulfate; scc, side-chain cleavage.

* Preliminary accounts of this work have appeared (30, 31).

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MATERIALS AND METHODS

Sodium cholate was obtained from Sigma and was recrystallized from water/acetone after charcoal treatment. Dithiothreitol and EDTA were also purchased from Sigma. Sepharose PD-10 columns, octyl-Sepharose 4B, Sephacryl 300, and blue dextran 2000 were obtained from Pharmacia. [26-^{14}C]Chol (59.6 mCi/mmol; 1 Ci = 3.7 × 10^{10} Bq) and tritiated water were purchased from New England Nuclear. [26-^{14}C]CholSO_4 was prepared and purified as described by Gasparini *et al.* (3). Bovine pancreatic trypsin was purchased from Worthington. Sucrose was ultrapure grade from Schwarz/Mann. Reagents for NaDodSO_4/polyacrylamide gels were purchased from Bio-Rad.

Purified adrenocortical mitochondrial scc enzyme (mitochondrial cytochrome P-450_{scc}) was purified as described by Greenfield *et al.* (6).

Adrenodoxin and Adrenodoxin Reductase. Adrenodoxin was purified by the method of Suhara *et al.* (15) with the modifications described by Greenfield *et al.* (6). Adrenodoxin reductase was purified by the procedure of Lambeth and Kamin (16).

Bovine Adrenocortical Cytosol. Bovine adrenal cortical tissue (350 g) was homogenized in 1 liter of 0.25 M sucrose/0.01 M potassium phosphate/1 mM dithiothreitol/1 mM EDTA, pH 7.4, as described. The post-mitochondrial supernatant was filtered through glass wool and centrifuged at 105,000 × *g* for 1 hr. The bright red supernatant (cytosol) was removed from the tubes with a syringe fitted with a plastic tip which was inserted through the lipid layer. Care was taken to avoid contamination from either the pellet or the lipid layer. The cytosol was stored at −20°C until used.

Preparation of the Modulator. All procedures were carried out at 4°C. Cytosol (250 ml, 10 mg of protein per ml) was applied to a 42 × 2.5 cm column of octyl-Sepharose 4B equilibrated in homogenization buffer. The column was washed with 800 ml of the same buffer until the absorbance of the wash at 280 nm was <0.05. Elution of the modulators was carried out with a 0.1 M potassium phosphate/0.25 M sucrose/1 mM dithiothreitol/1 mM EDTA, pH 7.4, containing 0.5% sodium cholate. Five-milliliter fractions were collected and the absorbance at 280 nm of each fraction was measured. Fractions absorbing light at this wavelength were pooled and concentrated on an Amicon PM-10 membrane under nitrogen pressure to a protein concentration of 10–20 mg/ml (fraction F). Before it was added to the scc assay system, fraction F was freed of cholate by passage through a PD-10 column of Sephadex G-25 equilibrated with 0.1 M Tris·HCl at pH 7.4.

Gel Filtration of Fraction F. A 5-ml sample of F to which 300,000 cpm of ^3H_2O and 0.2 ml of glycerol had been added was applied to a Sephacryl 300 column (85 × 1.5 cm) equilibrated in 0.01 M potassium phosphate/0.1 M KCl/1 mM dithiothreitol/1 mM EDTA, pH 7.4. The column was developed with the same buffer and 1-ml fractions were collected. Fractions were monitored for absorption of light at 280 nm and for their effects on the activity of the reconstituted scc system.

Assay of scc Activity. This activity was measured by using the assay of Hochberg *et al.* (17) with the modifications described by Greenfield *et al.* (6). Aliquots were taken at 3, 6, 9, and 12 min. Counts per minute were converted to molar concentrations of substrate and of isocaproic aldehyde products. K_m and V_{max} values were calculated from reciprocal plots by using the method of Lineweaver and Burk (18).

NaDodSO_4/Polyacrylamide Gel Electrophoresis. Electrophoresis was performed according to the method of Laemmli (19) on 15% polyacrylamide slab gels at 25 mA per gel. Gels were stained with silver reagent by the method of Merril *et al.* (20).

Protein Analysis. Protein concentrations were determined by the microbiuret method of Goa (21) with bovine serum albumin as a standard.

RESULTS

Detection of a Cytosolic Protein That Stimulates the scc of CholSO_4. In the course of this work we discovered that the 100,000 × *g* supernatant of homogenates of adrenal cortex had considerable scc activity. Fig. 1 depicts the effect of increasing the concentration of CholSO_4 and of Chol upon the rate of side-chain oxidation when the source of the cleavage enzyme was reconstituted scc system from mitochondria or from cytosol or a combination of these. In all cases, adrenodoxin, adrenodoxin reductase, and a NADPH-generating system were added to the assay systems. With the reconstituted enzyme system isolated from mitochondria, marked inhibition of activity was encountered at concentrations of CholSO_4 > 5 μM. The K_m was 0.3 μM and the V_{max} was 0.09 nmol/min per assay tube (0.01 nmol of enzyme). Similar results were obtained when Chol was used as the substrate: the K_m was 0.5 μM and the V_{max} of cleavage was 0.05 nmol/min per assay tube (0.01 nmol of enzyme). The enzyme system was also inhibited by Chol at >3 μM. Contrariwise, when cytosol was the source of the enzyme, there was no inhibition by substrate: oxidation of CholSO_4 occurred even when its concentration was as large as 60 μM. The K_m was

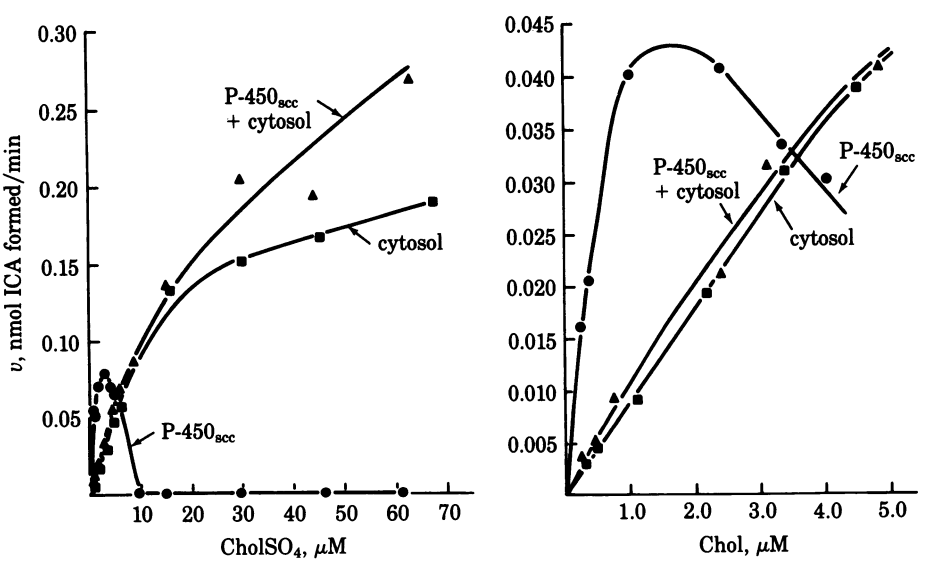

FIG. 1. scc of CholSO_4 (*Left*) and Chol (*Right*) by purified mitochondrial cytochrome P-450_{scc} (●) (0.01 nmol) or cytosol (0.2 ml, 2 mg of protein) (■), or a mixture of them (0.01 nmol of cytochrome P-450, 0.2 ml of cytosol containing 2 mg of protein) (▲). The reaction mixtures contained 1 nmol adrenodoxin, 0.1 nmol adrenodoxin reductase, and a NADPH-generating system in a total volume of 1 ml. ICA, isocaproic aldehyde.

23.2 μM and the V_{max} was 0.28 nmol/min per assay tube. For cholesterol the K_m was 16.5 μM and the V_{max} was 0.17 nmol/min per assay tube (2 mg of protein per ml).

The data shown in Fig. 1 also indicate that when cytosol and the purified mitochondrial cytochrome P-450$_{scc}$ were combined in the assay system, the rates of cleavage of substrates were not equal to the sum of the rates of cleavage when each enzyme source was assayed alone. At high concentrations of substrate, the mixture oxidized CholSO$_4$ at a rate greater than that with either enzyme source alone. At low concentrations of Chol and CholSO$_4$, the rates of cleavage by the mixture were lower than the sum of the individual activities. These observations suggested that there might be factors in the cytosol that affect the mitochondrial activity, inhibiting cleavage at low substrate concentrations and stimulating cleavage at high substrate concentrations.

Isolation and Characterization of a Modulatory Factor (Fraction F) from Cytosol. In order to search for the modulating factors in adrenal cytosol, it was chromatographed on the hydrophobic resin octyl-Sepharose. When the eluting solution was 0.5% sodium cholate in 0.1 M potassium phosphate at pH 7.4, a fraction (fraction F) was recovered that was devoid of scc activity. However, this fraction markedly altered the kinetics of oxidation of both CholSO$_4$ and CholSO$_4$ by mitochondrial cytochrome P-450$_{scc}$ (Fig. 2). With Chol as substrate, fraction F increased both the K_m and the V_{max} (for Chol, K_m = 3.5 μM, V_{max} = 0.08 nmol/min per assay tube). With CholSO$_4$ as substrate, fraction F increased only the K_m (for CholSO$_4$, K_m = 3.1 μM, V_{max} = 0.08 nmol/min per assay tube). In addition fraction F relieved the inhibition due to increasing concentrations of substrate; in the case of CholSO$_4$, the reversal of this inhibition was dramatic.

These effects on cleavage rates were destroyed by boiling fraction F for 2 min or by treating it with trypsin (data not shown).

Because fraction F had striking effects on the cleavage of CholSO$_4$ at both low and high concentrations of substrate (Fig. 2), 0.5 and 10 μM were chosen as convenient concentrations of CholSO$_4$ for the rapid detection of the modifying activity. The effects of adding increasing amounts of fraction F to the reconstituted mitochondrial scc system at these two concentrations of CholSO$_4$ are shown in Fig. 3. Fraction F has stimulatory activity at high concentrations of CholSO$_4$, with maximal stimulation at about 0.2 mg of protein. In contrast, the inhibitory activity evident at low concentrations of CholSO$_4$ appears not to have reached saturation even at 1.0 mg of added protein. These results suggested that two distinct modifiers were present in fraction F.

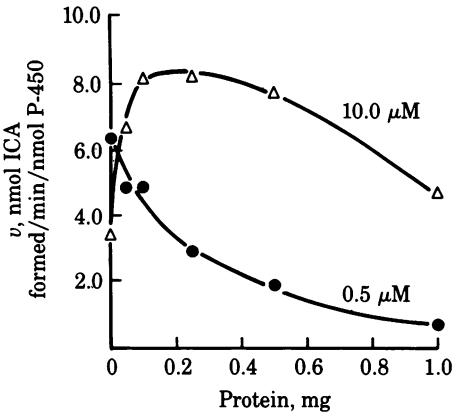

FIG. 3. Effect of increasing amounts of fraction F on the scc of CholSO$_4$ at 0.5 μM (●) and 10.0 μM (△) by purified mitochondrial cytochrome P-450$_{scc}$. Assay as in Fig. 1. ICA, isocaproic aldehyde.

Resolution of Fraction F into a Stimulator and an Inhibitor by Gel Filtration. In an effort to separate the stimulator and the inhibitor detected by the data shown in Fig. 3, fraction F was subjected to gel filtration on Sephacryl 300. Each point in Fig. 4 was determined by assaying the appropriate fraction in the reconstituted scc system with 1 μM Chol as substrate. Material that inhibited cleavage emerged from the column in the void volume. Although material that accelerated oxidation was eluted in many fractions, the peak of stimulatory activity was found in fractions 70–80, corresponding to species with molecular weights of <25,000. In a typical experiment, gel filtration of fraction F containing 50 mg of protein resulted in the recovery of 6 mg of protein in fractions 36–40 (F_I) and 13 mg of protein in fractions 59–90 (F_{II}). An attempt to concentrate F_I by using an Amicon PM-10 membrane under pressure failed because the material precipitated. F_I was used without further treatment in subsequent experiments.

Because the fractions containing the stimulator appeared to be stable to concentration by ultrafiltration, F_{II} was reduced in volume to a concentration of 4 mg of protein per ml. The con-

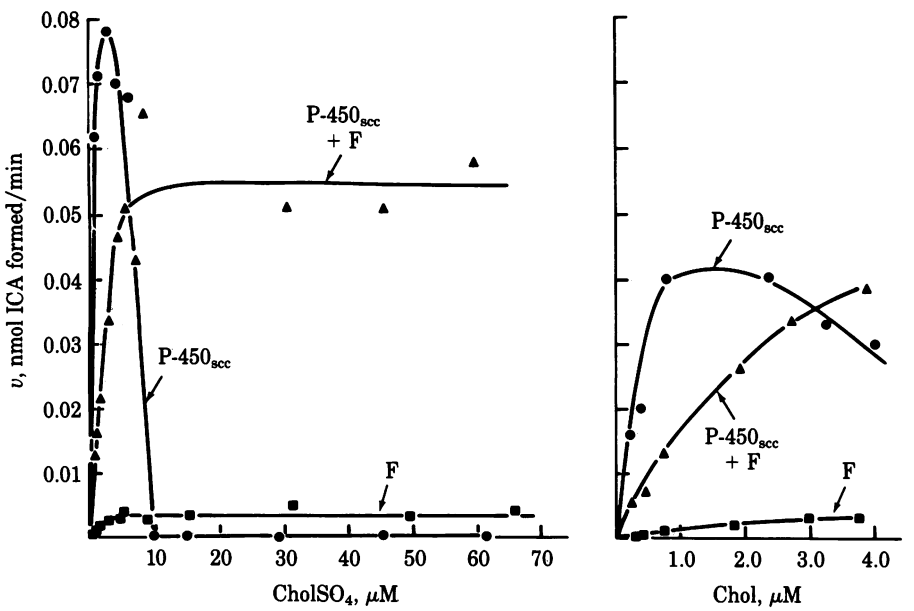

FIG. 2. scc of Chol and CholSO$_4$ by mitochondrial cytochrome P-450$_{scc}$ in the presence of fraction F. The conditions were: 0.01 nmol of cytochrome P-450 (●), fraction F from octyl-Sepharose chromatography (1 mg of protein) (■), or a mixture of them (0.01 nmol of cytochrome P-450 and 1 mg of protein) (▲). The conditions of assay were as in Fig. 1.

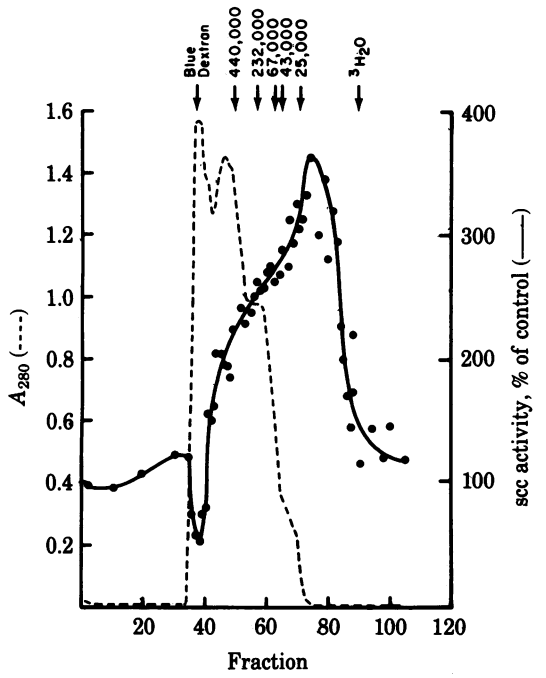

FIG. 4. Gel filtration of fraction F. A 100-μl sample of each fraction was incubated in a final volume of 200 μl for 3 min. Assay mixtures contained 2 pmol of cytochrome P-450, 0.02 nmol of adrenodoxin reductase, 0.2 nmol of adrenodoxin, and a NADPH-generating system.

centrated F_{II} and unconcentrated F_I were assayed in the reconstituted mitochondrial scc system with Chol and $CholSO_4$ as substrates. The data obtained were analyzed by the method of Lineweaver and Burk (Fig. 5). The kinetic constants calculated from the data in Fig. 5 are recorded in Table 1 as Exp. I. A duplicate experiment using cytosol from a second batch of bovine adrenals was carried out and these results are given as Exp. II. At two different concentrations (0.1 and 0.5 mg/ml) the inhibitory material increased the K_m for both Chol and $CholSO_4$ and decreased the V_{max} for $CholSO_4$. The stimulatory material increased the V_{max} of cleavage of both substrates but had little or no effect on the K_ms.

Incubation of the stimulatory material with trypsin destroyed its ability to affect scc activity (data not shown).

Gel electrophoresis was used to obtain an estimate of the molecular weight of the stimulator. The separate fractions resolved by gel filtration were subject to denaturing polyacrylamide gel electrophoresis. Every fifth fraction from fractions 55–90 (Fig. 4) was electrophoresed on a 5% polyacrylamide gel in the presence of $NaDodSO_4$; the gel was stained with silver reagent (Fig. 6). Although fractions 55–75 contained many proteins of high molecular weight, the only peptides detectable in fraction 80, which was within the peak of stimulatory activity, were of molecular weights <15,000.

Table 1. Effects of inhibitor and stimulator

Addition	Chol		$CholSO_4$	
	K_m, μM	V_{max}	K_m, μM	V_{max}*
	Exp. I			
None	1.7	3.5	0.5	8.7
F_I (0.1 mg)	4.0	4.5	2.5	4.6
F_{II} (0.5 mg)	1.2	11.2	0.6	13.0
	Exp. II			
None	1.2	2.7	0.1	4.2
F_I (0.5 mg)	8.6	2.4	6.1	1.2
F_{II} (0.5 mg)	2.7	9.6	0.9	5.0

* V_{max} is the maximal rate of scc expressed as mol of isocaproic aldehyde produced per min per mol of cytochrome $P\text{-}450_{scc}$.

DISCUSSION

Others (13, 14) have reported scc activity in cytosol from adrenal cortical tissue, but whether the active material in the cytosol is truly soluble or is merely a mitochondrial enzyme that dissociated from those organelles during homogenization was not determined unequivocally. It is evident (Fig. 1), however, that the K_ms of the activity in the cytosol for both Chol and $CholSO_4$ are more than an order of magnitude greater than those estimated when the reconstituted P-450 enzyme system isolated from the mitochondria was used.

The experiments described in this report demonstrate that the activity of a reconstituted sterol scc system can be modulated by at least two factors—an inhibitor and a stimulator—that are present in the cytosol of the adrenal cortex. These naturally occurring factors may serve to regulate substrate specificity of the cleavage enzyme system. If the observations described in this paper reflect the situation that prevails *in vivo*, they provide additional insight into possible mechanisms for the control

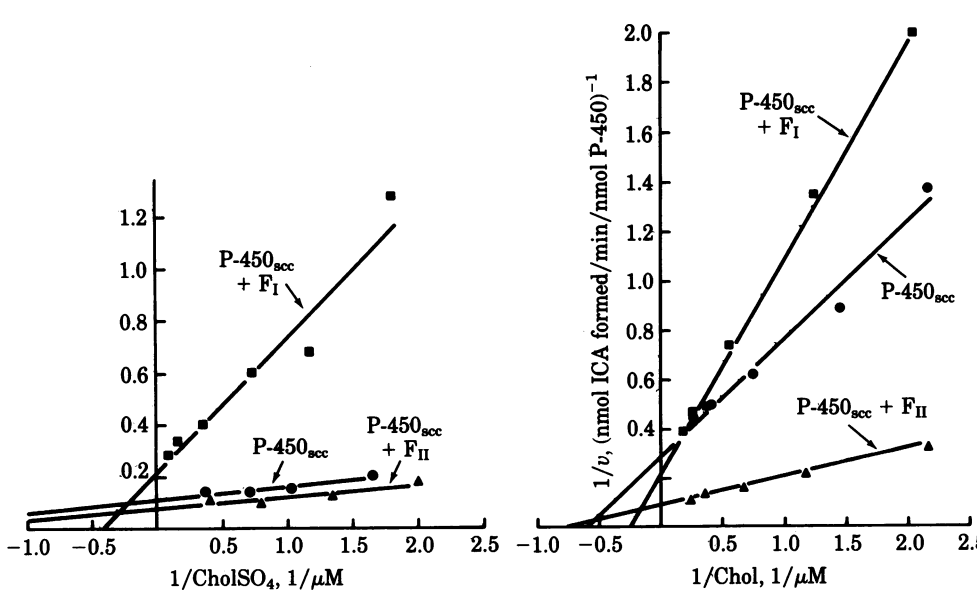

FIG. 5. Lineweaver–Burk analysis of scc of $CholSO_4$ (*Left*) and Chol (*Right*) by purified mitochondrial cytochrome $P\text{-}450_{scc}$ in the presence of inhibitor (F_I) and stimulator (F_{II}). Assay conditions as in Fig. 1.

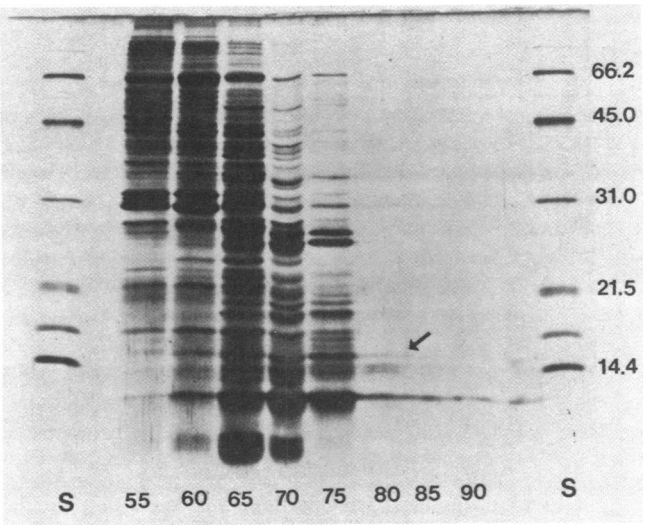

FIG. 6. $NaDodSO_4$/polyacrylamide gel electrophoresis of gel filtrates of fraction F. Twenty microliters of sample was used for each lane. The numbers under each lane refer to the fraction from which the material was obtained (see Fig. 4). S, lanes containing standard markers (sizes in kilodaltons): phosphorylase *b*, 92.5; bovine serum albumin, 66.2; ovalbumin, 45; carbonic anhydrase, 31; soybean trypsin inhibitor, 21.5; and lysozyme, 14.4.

of steroidogenesis through regulation of substrate utilization. In the absence of the modulatory factors, the maximal rate of $CholSO_4$ cleavage is more than 2-fold greater than that of Chol cleavage (Exp. 1 of Table 1). Addition of the inhibitor at 0.1 mg/ml depresses the V_{max} of $CholSO_4$ cleavage so that it is equal to the rate of Chol cleavage. Increasing the amount of inhibitory protein 5-fold (Exp. II) depresses $CholSO_4$ cleavage to half the rate of Chol cleavage. These results indicate that the inhibitory factor possesses substrate specificity. The inhibitor also increased the K_m of the reconstituted scc system for both substrates, as might be expected if it serves as a scavenger and sequesters substrate from the active site of oxidation.

The low molecular weight stimulator reported here increases the rate of Chol oxidation *in vitro* >3-fold but increases that of $CholSO_4$ oxidation only about 50%. Thus, the rate of cleavage of Chol becomes comparable to or even exceeds that of the more soluble $CholSO_4$. These results suggest that the stimulator functions as a solubilizer or carrier of the substrate. It is noteworthy that the activating factor does not increase the K_m of the enzyme system for Chol. This property seems to distinguish it from membrane phospholipids and nonionic detergents. These agents, which presumably mimic the natural membrane environment of the scc enzyme system, also increase the maximal rates of Chol cleavage in the reconstituted systems (22–26). However, phospholipids (26) and nonionic detergents (22–24) increase the K_m of the system for Chol significantly.

Protein synthesis is known to be necessary for the steroidogenic response to corticotropin in the adrenal gland (27, 28). In addition, adrenal cortical cells have been shown to respond to corticotropin with increased incorporation of [^{35}S]methionine into as yet unidentified cytoplasmic and mitochondrial proteins (29). The fact that the stimulator is of low molecular weight and is altered by tryptic digestion makes it a good candidate for a proteinaceous mediator that could be synthesized rapidly in response to tropic hormone stimulus.

For several decades, various steroidogenic enzyme deficiency diseases have been associated with genetic defects. 21-Hydroxylase and 5α-reductase are but two of the steroidogenic enzymes that are considered to be deficient in some hereditary diseases. For the most part, the deficiencies have been ascribed to the steroidogenic enzymes themselves. However, now that cellular proteins, such as those detected in this work, are known to be capable of regulating the activity of a steroidogenic enzyme, other loci vulnerable to faulty genetic expression need to be considered as etiological factors. In fact, these proteins may be invoked to explain the symptoms of some deficiency syndromes which have been attributed to "partial defects." In those situations in which an explanation involving complete enzyme blocks seems to be unsuitable, the inappropriate synthesis of naturally occurring regulatory proteins such as those reported in this paper may be related to the origin of such disorders.

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